Transcriptional Analysis of Vitiligo Skin Reveals the Alteration of WNT Pathway: A Promising Target for Repigmenting Vitiligo Patients

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Vitiligo affects 1% of the worldwide population. Halting disease progression and repigmenting the lesional skin represent the two faces of therapeutic challenge in vitiligo. We performed transcriptome analysis on lesional, perilesional, and non-depigmented skin from vitiligo patients and on matched skin from healthy subjects. We found a significant increase in CXCL10 in non-depigmented and perilesional vitiligo skin compared with levels in healthy control skin; however, neither CXCL10 nor other immune factors were deregulated in depigmented vitiligo skin. Interestingly, the WNT pathway, which is involved in melanocyte differentiation, was altered specifically in vitiligo skin. We demonstrated that oxidative stress decreases WNT expression/activation in keratinocytes and melanocytes. We developed an *ex vivo* skin model and confirmed the decrease activation of the WNT pathway in human skin subjected to oxidative stress. Finally, using pharmacological agents that activate the WNT pathway, we treated *ex vivo* depigmented skin from vitiligo patients and successfully induced differentiation of resident stem cells into pre-melanocytes. Our results shed light on the previously unrecognized role of decreased WNT activation in the prevention of melanocyte differentiation in depigmented vitiligo skin. Furthermore, these results support further clinical exploration of WNT agonists to repigment vitiligo lesions.

Journal of Investigative Dermatology (2015) **135,** 3105–3114; doi:10.1038/jid.2015.335; published online 24 September 2015

INTRODUCTION

Vitiligo is a disease of acquired depigmentation of the skin, sometimes affecting hair follicles. This condition affects 0.5–1% of the world population. Previous studies have clearly demonstrated that vitiligo greatly impairs the quality of life of affected individuals, and, as such, the development of an effective therapy is critical (Radtke *et al.*, 2009; Silverberg and Silverberg, 2013). The pathophysiology is complex and involves many cellular players. Both oxidative stress and the immune system have roles in vitiligo in genetically predisposed individuals (Jin *et al.*, 2012; Passeron and Ortonne, 2012; Spritz, 2012; Bellei *et al.*, 2013; Schallreuter *et al.*, 2013). One of the main challenges of studying vitiligo is that the affected cells, the melanocytes, are no longer present in the affected lesional skin. Animal models using reactive T cells against melanocyte antigens have provided interesting data about the immune reaction that is thought to be involved in the depigmentation of vitiligo skin, but these models are not adapted for studying the mechanisms of melanocyte differentiation and repigmentation in vitiligo skin (Mosenson *et al.*, 2013; Rashighi *et al.*, 2014). Although currently available treatments can provide cosmetically acceptable repigmentation (>75%; Taieb *et al.*, 2013), repigmentation consisting of the differentiation and proliferation of new melanocytes in depigmented vitiligo skin remains difficult to achieve in most cases.

In order to further characterize the pathophysiological mechanisms involved in vitiligo, we performed transcriptome (Affymetrix) analysis in combination with expression profiling of cytokines and chemokines in stratum corneum from lesional, perilesional, and non-depigmented skin samples from 10 vitiligo patients as well as from localization-matched skin samples from 10 healthy volunteers. Functional analyses were then performed to further explore our findings using *in vitro* experiments and an *ex vivo* skin model of vitiligo.

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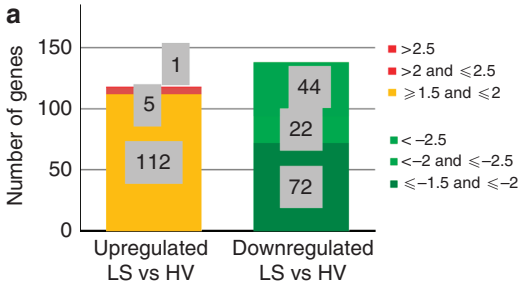
Received 7 April 2015; revised 1 July 2015; accepted 19 July 2015; accepted article preview online 31 August 2015; published online 24 September 2015

RESULTS

Transcriptome analysis revealed a low-level immune reaction in non-depigmented and perilesional vitiligo skin and repression of the WNT pathway in depigmented skin

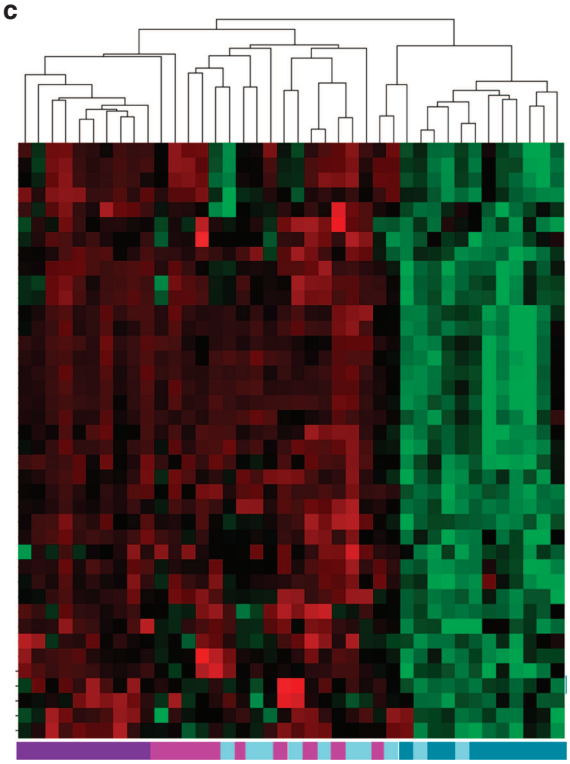
A total of 118 genes were upregulated, and 138 were downregulated in lesional vitiligo skin compared with the skin from healthy controls (Figure 1a). When perilesional and non-depigmented vitiligo skin samples were compared with samples from healthy controls, 110 and 98 annotated genes were found to be upregulated, and 21 and 18 annotated genes were downregulated, respectively. Ingenuity pathway analysis of overlapping canonical pathways revealed significant deregulation of melanocyte development and pigmentation signaling, circadian signaling, and the WNT/β-catenin pathway in vitiligo lesions compared with control skin (Figure 1b). Hierarchical clustering of genes that were differentially expressed between lesional and healthy skin from all samples highlighted a transcriptional signature of melanocyte loss with an almost complete extinction of the expression of melanocyte markers in lesional samples (Figure 1c). Although we sought to identify modulations of genes reported to be expressed in various immune cell subsets, we observed very few variations among the vitiligo samples, and none reached the level of statistical significance (Supplementary Table S1 online).



b

Ingenuity canonical pathways	*P*-value	Count	Genes
Melanocyte development and pigmentation signaling	4.5E–07	9	*TYRP1*, *ADCY2*, *MITF*, *TYR*, *SOX10*, *PAX3*, *KIT*, *DCT*, *MC1R*
Circadian rhythm signaling	1.7E–06	6	*PER3*, *PER1*, **ARNTL**, *NR1D1*, *BHLHE41*, **CLOCK**
Wnt/β-catenin signaling	3.2E–03	7	**TP53**, *CDH2*, *CDH3*, *SOX10*, **DVL1**, **TLE4**, *LEF1*

c



Gene symbol	Probeset	FC	FDR adj *P* value
GPM6B	209168_at	−3.4	3.6E−06
GPM6B	209167_at	−3.5	1.2E−06
GPM6B	209170_s_at	−3.7	3.0E−06
GPM6B	209169_at	−3.2	6.1E−07
IRF4	204562_at	−3.1	9.9E−06
ARNT2	202986_at	−2.2	2.2E−03
SLC45A2	220245_at	−2.3	1.2E−03
CA14	219464_at	−4.4	1.1E−06
CAPN3	210944_s_at	−3.4	4.6E−07
CAPN3	211890_x_at	−3.3	6.9E−06
CAPN3	214475_x_at	−3.6	8.4E−07
DCT	216512_s_at	−17.2	2.9E−07
MYEF2	222153_at	−18.2	1.6E−06
DCT	205338_s_at	−41.8	5.0E−07
DCT	205337_at	−30.5	1.2E−06
MLANA	206426_at	−36.6	1.2E−06
MLANA	206427_s_at	−24.3	6.5E−07
TYR	206630_at	−21.6	4.5E−07
TYRP1	205694_at	−27.2	1.4E−05
PMEL	209848_s_at	−6.6	1.3E−05
TYR	1555505_a_at	−14.1	1.8E−06
TRPM1	206479_at	−7.6	4.0E−07
OCA2	206498_at	−4.1	9.1E−06
PCSK2	204869_at	−6.8	1.6E−06
PCSK2	204870_s_at	−4.9	3.2E−06
GPR143	206696_at	−6.4	3.2E−06
VEPH1	232122_s_at	−7.0	3.6E−06
INPP4B	235046_at	−2.0	5.2E−03
MYEF2	222771_s_at	−3.7	2.4E−05
MYEF2	232676_x_at	−2.8	3.0E−04
PLP1	210198_s_at	−3.4	1.3E−05
APBA2	209871_s_at	−2.6	1.2E−03
LPPR4	213496_at	−3.5	6.5E−05
TRIM63	236972_at	−2.0	9.3E−03
TRPM1	237069_s_at	−4.3	2.2E−05
TRPM1	237070_at	−10.0	3.4E−06
CRYM	205489_at	−2.2	2.0E−04
RNF144A	204040_at	−1.8	1.0E−04
MITF	207233_s_at	−1.7	3.0E−04
MITF	226066_at	−1.8	2.0E−04

Figure 1. Transcriptomic analysis of vitiligo patients highlights the disappearance of melanocytes and the involvement of the WNT signaling pathway in the absence of immune system activation. (**a**) Histogram of differentially expressed genes between lesional and healthy samples depicts a similar number of significantly upregulated and downregulated genes. (**b**) Ingenuity-based canonical pathways that were enriched in vitiligo lesional skin. Genes highlighted in bold are upregulated, and those in italics were downregulated. (**c**) Cluster analysis of the microarray data from healthy skin (HS), non-depigmented non-lesional skin (NLS), perilesional skin (PLS), and lesional skin (LS) from vitiligo subjects. All probes significantly modulated between HS and LS samples ($|FC| \geq 1.5$ with a FDR < 0.05, $n = 333$) were included in the cluster analysis, and the transcriptional signature corresponding to melanocyte loss was the focus. Gene symbol, probe set number, fold change between LS and HS samples, and FDR-adjusted *P*-values of melanocytic genes are presented in the accompanying table. FDR, false discovery rate.

Interestingly, a transcriptional network linking WNT signaling and melanogenesis pathways was observed using ingenuity pathway analysis (Supplementary Figure S1c online). Lesional vitiligo skin is characterized by downregulated expression of lymphoid enhancer binding factor 1 (LEF1), the key transducer of the WNT signaling pathway, and of downstream effectors, such as cadherin 2 and cadherin 3 and INF regulatory factor 4 (IRF4). Lesional skin is also characterized by upregulation of negative regulators of the WNT signaling pathway, such as p53, which is involved in signal transduction of WNT players; TLE4, a Groucho family member; and ZBTB33/Kaiso, which is involved in the transcriptional repression of WNT target genes. Furthermore, the transcription factor LEF1 directly induces expression of microphthalmia-associated transcription factor (MITF), which accordingly is downregulated in vitiligo skin. IRF4 has recently been identified as a direct target of LEF1 and MITF. At the functional level, IRF4 transactivates MITF function by binding to shared melanocytic gene promotors (Praetorius *et al.*, 2013). Furthermore, LEF1 and β-catenin regulate melanogenesis through SOX10 transcriptional induction, which in turn regulates the expression of the EDNRB, PCSK2, and PLP1 genes.

Our transcriptional analysis also revealed modulation of the circadian pathway in the vitiligo samples. Because of the known variation of these genes during the day, we investigated whether these modulations were due to the time of sampling or were linked directly to vitiligo pathogenesis. We analyzed the expression of the key circadian genes (ARNTL/BMAL1, CLOCK, PER1, and NR1D1) with respect to the hour when the samples were taken. A strong correlation was found between the hour of sampling and gene expression, indicating that modulation of expression of these circadian genes was not linked to vitiligo pathology but rather to sequential biopsy sampling at 1-h intervals (Supplementary Figure S2a online). Following synchronization of melanocytes, LEF1 expression was not correlated with ARNTL expression (Supplementary Figure S2b online), and this finding was confirmed at the messenger RNA (mRNA) level for several WNT members examined during the course of the circadian cycle (data not shown).

The results of the transcriptional analysis were then examined with quantitative real-time reverse-transcriptase–PCR using Taqman Low Density Array (Life Technology, Carlsbad, CA, USA). A marked decrease in expression of all the melanocytic genes in vitiligo skin compared with that in control skin confirmed the loss of melanocytes in the affected vitiligo skin (Figure 2a). Analysis of the expression of LEF1, which serves as a key marker of the activation of the WNT pathway, revealed that this factor was downregulated in lesional vitiligo skin, in accord with the results of the transcriptional analysis (Figure 2b). Finally, because CXCL10 has been implicated in a mouse model of vitiligo (Rashighi *et al.*, 2014), we also analyzed CXCL10 expression in our skin samples using Taqman Low Density Array. We observed a significant increase in CXCL10 expression in perilesional skin but also in non-depigmented skin of vitiligo patients compared with healthy controls; however, the level of expression of CXCL10 in depigmented vitiligo lesions was not different than that in healthy skin (Figure 2c).

These results indicated insignificant modulation of genes involved in the immune reaction, although CXCL10 was significantly upregulated in perilesionaland in non-depigmented skin of vitiligo patients. Thus, these findings highlighted the absence of an immune reaction in lesional skin devoid of melanocytes and interestingly revealed a downregulation of the WNT/β-catenin pathway in lesional vitiligo skin.

Analysis of stratum corneum cytokines shows no significant dysregulation in vitiligo skin

A total of 62 cytokines were examined in the stratum corneum of subjects with vitiligo and healthy volunteers via tape stripping. Twelve cytokines were detected and quantified with concentrations ranging from 1 to 7,000 pg mg^{-1} of protein (Supplementary Table S2 online). No significant difference in the cytokine profiles between pathological and non-pathological samples was observed (Supplementary Table S3 online). The concentration of CXCL10 protein in the stratum corneum was compared with the mRNA expression obtained from skin biopsies. Although CXCL10 was found to be significantly upregulated within the non-lesional and perilesional vitiligo skin compared with healthy controls at the mRNA level, no significant modulation was observed at the protein level in the stratum corneum between these samples (Supplementary Table S4 online). These results demonstrate that cytokines do not accumulate in the stratum corneum of lesional skin and that the immune reaction in vitiligo therefore occurs at a very low level and is no longer detectable in depigmented skin regions.

Oxidative stress decreases WNT pathway activity in the skin

As the WNT pathway appeared to be affected in vitiligo skin, we sought to determine the specific factor responsible for this dysregulation. Oxidative stress has been reported to inhibit the WNT/β-catenin pathway in kidney cells (Shin *et al.*, 2004), and other studies support a role for oxidative stress in vitiligo (Bellei *et al.*, 2013; Schallreuter *et al.*, 2013). We, therefore, examined the impact of oxidative stress on WNT/β-catenin activation in an *ex vivo* skin model. H_2O_2 decreased the expression of LEF1 and CDH3 and of most WNT family members, whereas the level of β-catenin remained stable (Figure 3a and Supplementary Figure S3c online). H_2O_2 also decreased the expression of the LEF1 and WNT family members in melanocyte and keratinocyte cultures (Figure 3b) and the activity of the TCF/LEF promoter in melanocyte culture (Figure 3c). In agreement to the results obtained with the transcriptome analysis, after 8 days of treatment with H_2O_2, LEF1 and CDH3 remained decreased in *ex vivo* skin, whereas the WNT members tended to exhibit increased expression (Figure 3d and Supplementary Figure S3d online).

Development of an *ex vivo* model to study vitiligo skin

The difficulty in studying vitiligo lies in the absence of a model that mimics the *in vivo* conditions and also contains stem cells that could be targeted to induce repigmentation.

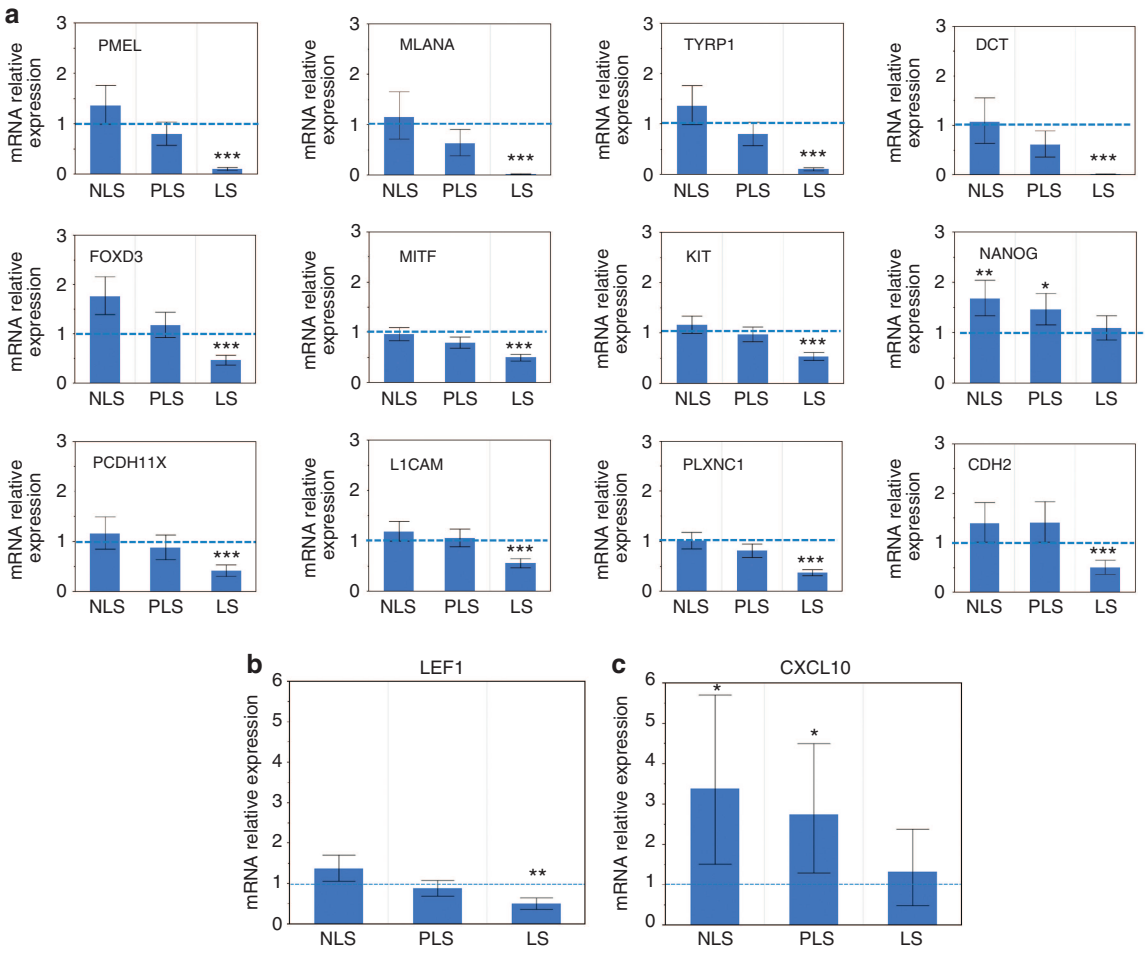


Figure 2. Gene expression analysis of the key factors emphasized by the transcriptomic analysis. Gene expression was measured by quantitative real-time reverse-transcriptase–PCR of (**a**) melanocytic genes, (**b**) LEF1, and (**c**) CXCL10 mRNA. Non-lesional skin (NLS), perilesional skin (PLS), and and lesional skin (LS) sample groups were compared with the healthy skin (HS) group using a *t*-test linear model without pairing. Differences in gene expression were considered significant if both the lfold changel was ⩾1.8 and the FDR value was <0.05. The *y* axis displays the fold change in expression relative to the pool of healthy skin samples. The data were normalized relative to expression of HPRT, ACTB, and GAPDH. The error bars display the SE of three replicates. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. The TLDA validation set encompassed genes related to terminal differentiation of melanocytes (PMEL, MLANA, TYRP1, and DCT), transcription factors involved in melanogenesis (MITF, FOXD3, KIT, and NANOG), and adhesion molecules expressed in melanocytes (PLXNC1, PCDH11X, CDH2, and L1CAM). CDH2, cadherin 2; DCT, dopachrome tautomerase; FDR, false discovery rate; mRNA, messenger RNA; LEF1, lymphoid enhancer binding factor 1; MITF, microphthalmia-associated transcription factor; TLDA, Taqman Low Density Array; TYR, tyrosinase.

Thus, we developed an *ex vivo* skin model that is viable long enough to induce the differentiation of melanocyte stem cells in vitiligo skin. In clinical practice, vitiligo lesions usually require months to achieve complete or almost complete repigmentation; however, in the best cases, the onset of pigmentation can sometimes be observed after 15 days of treatment. We therefore examined the morphologic characteristics of skin after treatment with the pigmentation inducer forskolin after 15 days of *ex vivo* culture following abdominoplasty skin surgery. At this time, the morphology of the skin remained stable, compared with the initial conditions. We noted only a flattening of the dermal–epidermal junction, and such flattening may be owing to a difference of tension in the skin (Figure 4a). The ability of the skin to respond to forskolin was analyzed by quantitative PCR and immunofluorescence by studying the expression of the melanogenic genes MITF, dopachrome tautomerase (DCT), and tyrosinase. In order to assess the dose response of forskolin, the skin was stimulated by forskolin systemically every other day. As expected, the initial response induced upregulation of MITF mRNA (Figure 4b) and protein (Figure 4c) after 11 days of stimulation. This phenomenon was transient, although expression remained high after 15 days at a low forskolin concentration (Figure 4b). After 15 days, we observed a strong increase of the melanogenic enzymes DCT and tyrosinase at both the mRNA and protein levels (Figures 4b and c). Despite the activation of the melanogenesis pathway, we did not detect an increase in melanin contain in the cells (data not shown). In conclusion, our model of *ex vivo* skin culture was viable and functional for as many as 15 days in culture.

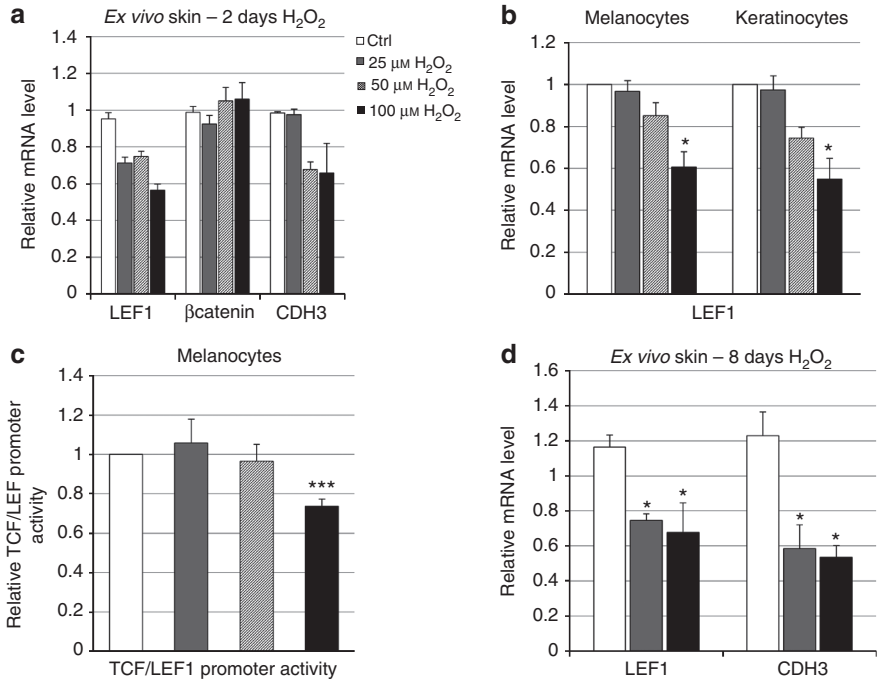


Figure 3. Oxidative stress decreases WNT pathway activity in the skin. In whole skin in *ex vivo* culture, NHM and NHK were stimulated for 24 h (**b**), 2 days (**a**), or 8 days (**d**) with 25–100 μM H_2O_2. After mRNA extraction, reverse transcription was performed, and the relative gene expression of LEF1, β-catenin, and CDH3 was analyzed using quantitative PCR. Results were normalized with the expression of SB34.(**c**) NHM were infected with lentivirus luciferase TCF/LEF1 reporter. After selection, NHM were treated for 24 hours with H_2O_2, and the relative activity of TCF/LEF was quantified with a firefly luciferase assay and normalized with Renillaluciferase expression. *$P<0.05$; **$P<0.01$, ***$P<0.001$. mRNA, messenger RNA; LEF1, lymphoid enhancer binding factor 1.

Pharmacological WNT pathway activators induce increased WNT pathway expression in *ex vivo* cultured vitiligo skin

The WNT pathway is involved in melanocyte differentiation (Yamada *et al.*, 2013; Fukunaga-Kalabis *et al.*, 2015), and we have demonstrated that this pathway is altered in vitiligo skin. In order to induce the differentiation of melanocyte stem cells, we pharmacologically activated the WNT pathway in *ex vivo* vitiligo skin using the WNT agonist (SKL2001) and the glycogen synthase kinase (GSK)3β inhibitors lithium chloride (LiCl) and CHIR99021. We obtained biopsies from nine vitiligo subjects from diverse body locations (three from the elbow, two from the trunk, two from the leg, one from the arm, and one from the axilla). We then treated the *ex vivo* cultures for 14 days via systemic stimulation every other day with these WNT activators. Evaluation of the activation of the WNT pathway indicated that treatment with LiCl, CHIR99021, or SKL2001 induced all WNT members and LEF1 at the mRNA level after 14 days of stimulation (Figure 5a and Supplementary Figure 4 online).

Ex vivo treatment of depigmented skin biopsies from vitiligo patients with WNT activators induces the differentiation of resident stem cells into pre-melanocytes

As we succeeded in upregulating the WNT pathway in the *ex vivo* vitiligo skin biopsies, we analyzed the expression of melanoblast markers following treatment. The mRNA levels of early melanoblast markers, such as PAX3 and BRN2, were upregulated in biopsies stimulated with CHIR99021, SKL2001, and LiCl (Figures 5b and c). This result suggests initiation of melanocyte differentiation. MITF levels were increased only in the skin treated with SKL2001 (Figure 5d); however, this expression was transient following stimulation, suggesting that the time response is different for the different treatments. The pre-melanocyte marker DCT was increased in response to all treatments, but this effect was stronger in response to LiCl (Figure 5e).

We next employed immunofluorescence to study the co-expression of DCT and PAX3 in order to investigate whether these treatments lead to the differentiation of melanocyte stem cells in pre-melanocytes in the skin. In vitiligo skin biopsies cultured under control conditions, we observed no or few isolated cells expressing PAX3 and DCT (Figure 5f). Following stimulation with Wnt activators (Figures 5g–i), we found many clusters of cells co-expressing the two markers in the hair follicles and in the dermis, representing melanocytes undergoing differentiation. Taken together, these results showed that targeting the deficient WNT pathway of vitiligo skin using WNT agonists or GSK3β inhibitors leads to the differentiation of melanocyte stem cells into pre-melanocytes.

DISCUSSION

Our results emphasize the complexity of vitiligo pathophysiology. Although these findings partially support the role of the immune system, and notably CXCL10, in depigmentation of the skin in vitiligo patients, these data also indicate that neither CXCL10 nor other immune factors are deregulated in

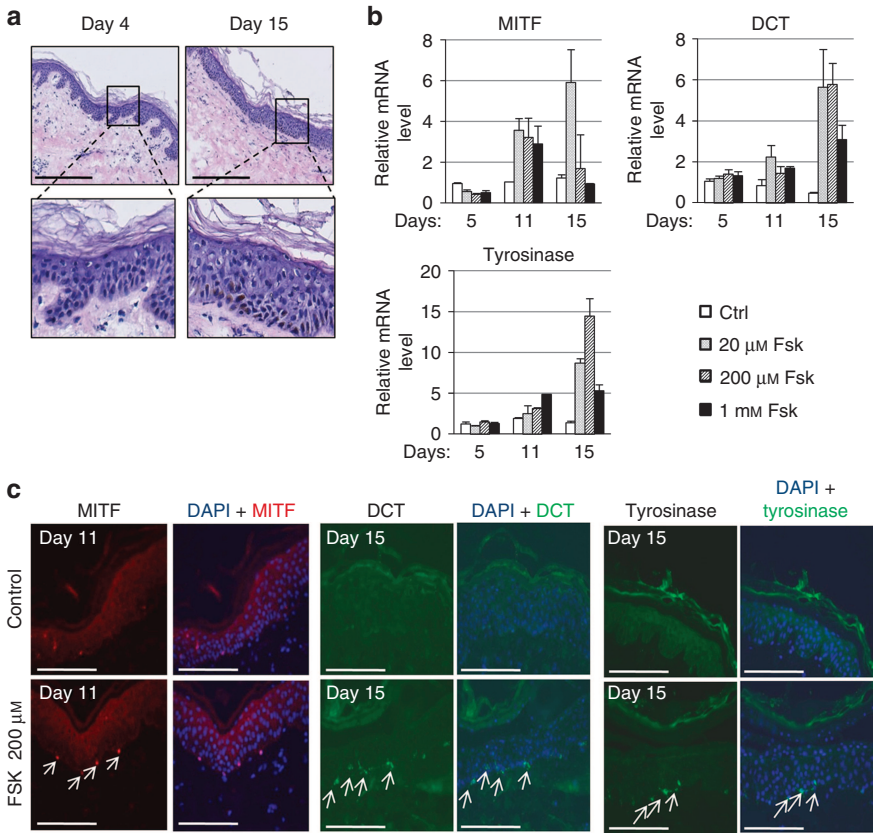


Figure 4. Development of an *ex vivo* skin model to study vitiligo skin. Skin biopsies (6 mm) were obtained from abdominoplasty surgery and disposed on a Transwell chamber to allow a semi-liquid culture environment. The skin was stimulated with 20 μM to 1 mM forskolin supplemented in the culture medium every second day from day 4 to day 15. (**a**) After 4, 11, and 15 days, the morphology of the skin was visualized by microscopy following hematoxylin and eosin staining (scale bar = 300 μm). (**b**) The ability of the skin to respond to melanogenic stimuli was assessed using forskolin treatment. The mRNA was extracted from biopsies and analyzed by quantitative real-time reverse-transcriptase–PCR to quantify the expression of the melanogenic genes MITF, DCT, and TYR. (**c**) Immunohistochemical staining was performed to study the expression of MITF, DCT, and TYR proteins after 11 or 15 days of forskolin stimulation (scale bar = 100 μm). DCT, dopachrome tautomerase; mRNA, messenger RNA; MITF, microphthalmia-associated transcription factor; TYR, tyrosinase.

already depigmented vitiligo skin. Recently, the role of CXCL10 and the INF-γ pathway in a vitiligo mouse model and in some human vitiligo skin samples was demonstrated (Harris *et al.*, 2012; Rashighi *et al.*, 2014). Interestingly, the samples analyzed were selected because of the presence of an immune infiltrate. Our results, which show an increase in CXCL10 expression in perilesional skin compared with healthy control skin, are consistent with these data; however, we also discovered that CXCL10 expression is significantly increased within unaffected skin of vitiligo patients as compared with healthy control skin, suggesting that even non-depigmented skin in vitiligo patients has low-level immune activation that culminates in CXCL10 expression. On the contrary, we did not observe any increase in the CXCL10 expression in depigmented lesions that lack melanocytes. CXCL10 upregulation in non-lesional and perilesional skin may indicate a constitutive level of secretion by vitiligo skin cells upon stress signaling. Indeed, CXCL10 is induced by a variety of innate stimuli that induce IFN-α/β as well as the adaptive immune cell cytokine IFN-γ. CXCL10 is best known for its role in leukocyte trafficking, primarily acting on CXCR3-expressing $CD4^+$ Th1 cells, $CD8^+$ T cells, and natural killer cells. Therefore, we speculate that skin-resident memory T cells and/or melanocytes themselves may serve as the initial CXCL10 producers, ultimately inducing the recruitment of $CXCR3^+$ cells. Once melanocyte destruction is complete and the immune reaction is no longer active, CXCL10 is no longer produced in the lesional areas. Interestingly, we recently demonstrated in a prospective randomized placebo-controlled study that 0.1% tacrolimus ointment applied twice weekly significantly lowers the relapse rate of successfully repigmented vitiligo lesions (Cavalie *et al.*, 2014). The efficacy of this maintenance therapy strongly suggests that a low-level immune reaction occurs in the pigmented skin of vitiligo patients. The only other transcriptome analysis performed in vitiligo skin before now failed to reveal potent activation of the immune system, although in contrast to our findings, this previous study pointed to activation of some factors involved in innate immunity (in particular, on natural killer cells; Yu *et al.*, 2012). Although we ensured that the lesions were clinically active, we cannot rule out the possibility that the samples in the perilesional skin were devoid of immune infiltrate. This may have prevented the detection of immune dysregulation (aside

from CXCL10) and may also explain the fact that we did not observe activation of natural killer cells as previously shown by Yu *et al.* (2012).

Recently, the WNT/β-catenin pathway was found to have a key role in UVB-induced melanocyte stem cell differentiation (Yamada *et al.*, 2013). Within the skin, the secretion of WNT

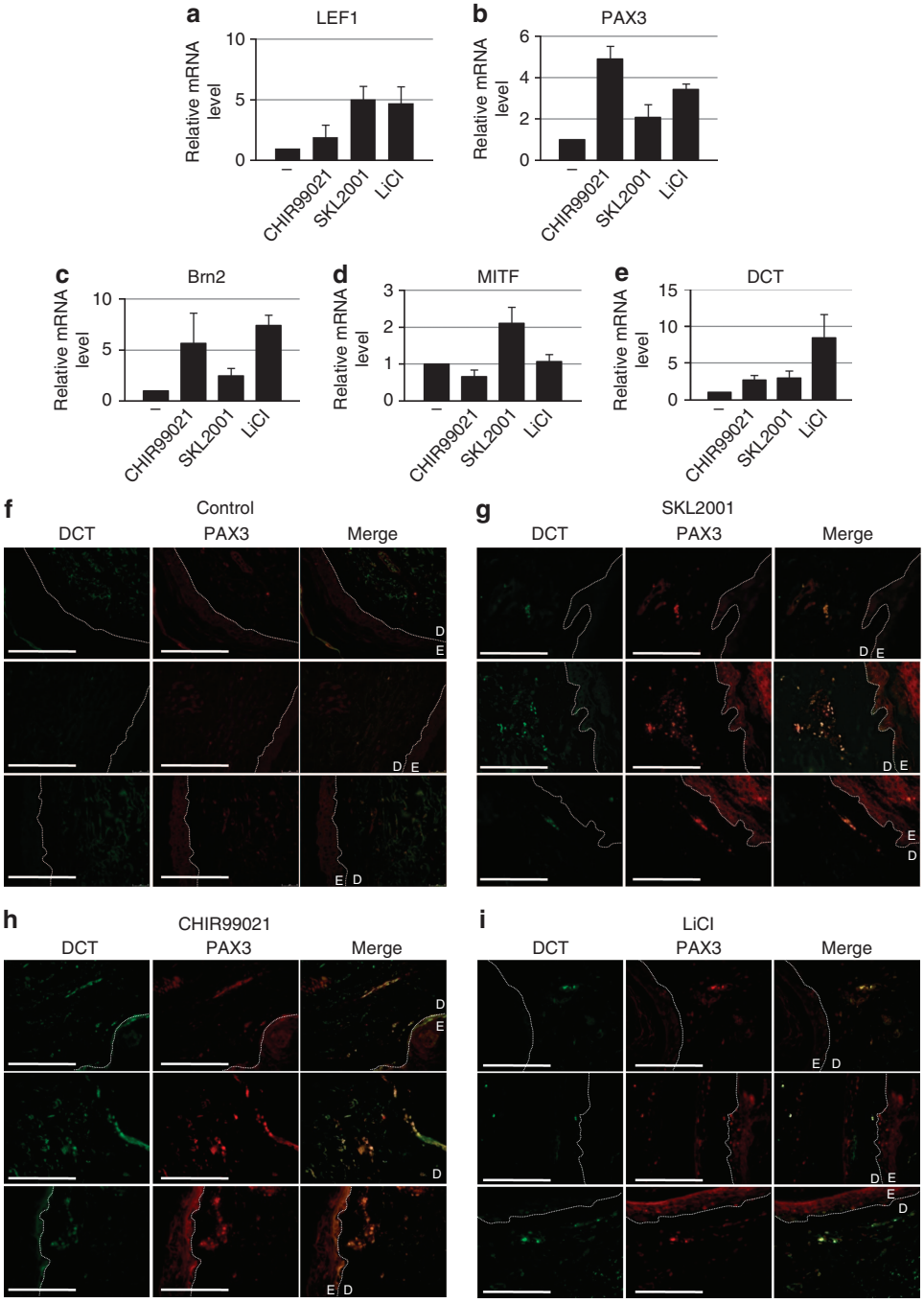


Figure 5. Treatment of *ex vivo* depigmented skin samples from vitiligo patients with pharmacological WNT pathway activators induces the differentiation of resident stem cells into pre-melanocytes. Lesional skin from vitiligo patients ($n = 9$) was biopsied. The biopsies were stimulated in *ex vivo* culture for 14 days with the GSK3β inhibitors CHIR99021 (3 μM) or LiCl (20 μM) or with the WNT agonist SKL2001 (40 μM) every other day. Then, mRNA was extracted, and quantitative real-time reverse-transcriptase–PCR was performed to quantify the relative expression of LEF1 (**a**) and the melanocyte markers PAX3 (**b**), BRN2 (**c**), MITF (**d**), and DCT (**e**). The response of the vitiligo skin was analyzed by immunohistochemical staining for the pre-melanocyte markers DCT (green) and PAX3 (red). The co-localization (yellow) observed on merge pictures shows melanoblasts in differentiation within the dermis following treatment with the following agents: (**f**) control, (**g**) WNT agonist SKL2001 (40 μM), (**h**) CHIR99021 (3 μM), and (**i**) LiCl 20 μM (scale bar = 100 μm). D, dermis; DCT, dopachrome tautomerase; E, epidermis; LEF1, lymphoid enhancer binding factor 1; LiCl, lithium chloride; MITF, microphthalmia-associated transcription factor; mRNA, messenger RNA.

mainly by keratinocytes and melanocytes contributes to the differentiation of stem cells in melanocytes. Our transcriptional analysis reveals an alteration of the WNT/β-catenin pathway in vitiligo skin with a significant decrease in LEF/TCF expression that was confirmed using quantitative real-time reverse-transcriptase–PCR. As LEF1 is predominantly expressed by melanocytes in the skin, we cannot exclude the possibility that this decrease merely reflects the absence of melanocytes; however, other WNT pathway genes that were found to be differentially expressed in the transcriptome analysis are similarly expressed in keratinocytes and melanocytes (e.g., CDH3, TLE4, and Kaiso; Supplementary Figure 5 online). In addition, our functional analyses further support the impact of the decreased activation of the WNT pathway in vitiligo as decreased expression of LEF1 and decreased activity of the LEF1/TCF promoter were observed after oxidative stress and differentiation of resident stem cells was induced in pre-melanocytes following *ex vivo* stimulation with WNT activators.

Our transcriptome analysis also demonstrated that adhesion proteins, including cadherins, were also decreased in vitiligo skin. The WNT pathway is known to regulate E-cadherin expression, and interestingly, recent data showed decreased expression of E-cadherin across melanocyte membranes in vitiligo patients, leading to decreased adhesiveness of these cells to the basal layer under oxidative and mechanical stress (Wagner *et al.*, 2015). Many studies have emphasized the role of oxidative stress in vitiligo (Maresca *et al.*, 1997; Bellei *et al.*, 2013; Schallreuter *et al.*, 2013). Furthermore, a link between oxidative stress and activation of the immune response has recently been uncovered (Passeron and Ortonne, 2012; Toosi *et al.*, 2012), and our data clearly indicate that oxidative stress decreases WNT pathway activity in melanocytes and keratinocytes. WNT ligands and LEF1 are first decreased in both melanocytes and keratinocytes; yet, decreased levels of LEF1 and CDH3 persist in vitiligo skin *ex vivo* despite the augmentation of WNT probably owing to compensation of the impaired pathway. In vitiligo lesions devoid of melanocytes, the keratinocytes are presumably responsible for the production of WNT proteins. Taking into account the central role of the WNT/β-catenin pathway on the differentiation of melanocyte stem cells, we hypothesize that oxidative stress negative impacts the differentiation of melanocytes in vitiligo skin. We, therefore, directly addressed the defective differentiation of melanocyte stem cells by stimulating the WNT/β-catenin pathway, which was altered in vitiligo lesions. Using our *ex vivo* model for depigmented skin of vitiligo patients, we demonstrated that treatment with WNT agonists or GSK3β inhibitors induce increased expression of melanocyte markers, triggering the differentiation of resident melanocyte stem cells in pre-melanocytes expressing PAX3 and DCT. Interestingly, we observed pre-melanocytes not only in the hair follicles but also in the dermis, suggesting that this approach may be helpful for the differentiation of dermal stem cells of glabrous skin (Li *et al.*, 2010). The cells that expressed DCT and PAX3 remained in the dermis, and we did not detect the differentiated melanocyte marker tyrosinase at either the mRNA or protein level (using immunohistochemistry) in these cells (data not shown). Such localization and expression patterns of melanocyte markers strongly suggest that these cells are pre-melanocytes. In all likelihood, however, the limited timeframe of our *ex vivo* model is too short to obtain fully differentiated melanocytes.

Taken together, our results demonstrate that the immune reaction in vitiligo occurs only at very low levels. Specifically, an increase in CXCL10 expression in non-depigmented and perilesional skin was observed, and an immune reaction is no longer detectable in vitiligo lesions already devoid of melanocytes. These findings also highlight a previously unrecognized defect in WNT/β-catenin activation triggered by oxidative stress, and this defect may effectively prevent the differentiation of melanocyte stem cells (Figure 6). These results not only provide a better understanding of the complex pathophysiology of vitiligo but also support further clinical exploration of WNT activators for repigmenting vitiligo lesions.

MATERIALS AND METHODS

Patients for transcriptomic analysis

Ten patients with active non-segmental vitiligo, which is defined by the occurrence or the worsening of depigmented lesions in the past 3 months and having hypochromic borders upon Wood's lamp examination, were enrolled in the study after informed, written consent was obtained. The study was approved by the local ethics committee (N12.034). A 4-mm-skin biopsy was taken from each patient in the center of a vitiligo patch, in the perilesional area (defined as 5 mm outside of the lesion border), and in non-lesional skin located in the same area but at least 3 cm from a depigmented lesion. A 4-mm biopsy was also taken from 10 healthy patients and served as a control that was matched for gender, age, and location. The procedure for taking samples and the characteristics of the patient population are described in Supplementary Figures S1a and S1b online.

Transcriptomic analysis

Biopsies for the microarray analysis were stored in RNA Stabilization Reagent (Qiagen, Venlo, The Netherlands) until use. For RNA extraction, the samples were homogenized with a potter in Qiagen lysis buffer (Qiagen). Total RNA was extracted using RNeasy extraction kits (Qiagen) according to the manufacturer's protocol. RNA quantity was measured using a Nanodrop Spectrophotometer ND8000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality was monitored using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Probes were synthesized and then hybridized on Affymetrix U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA, USA). All chips were normalized using the robust multi-array average method (Bolstad *et al.*, 2003). Only Affymetrix identifiers (IDs) with expression $\geqslant 2\exp 6(64)$ for at least 7 out of 10 samples in at least 1 sample group (lesional (LS), non-lesional (NLS), perilesional (PLS), or healthy) were selected. Finally, 29,906 of 54,675 IDs that were initially present were kept for statistical analyses, and thresholds of modulation of 1.5 and of -1.5 were selected for further analyses. Data analysis was performed on Array Studio software (OmicSoft, Cary, NC, USA). A two-sided paired Student's *t*-test was performed. The Benjamini-Hochberg procedure

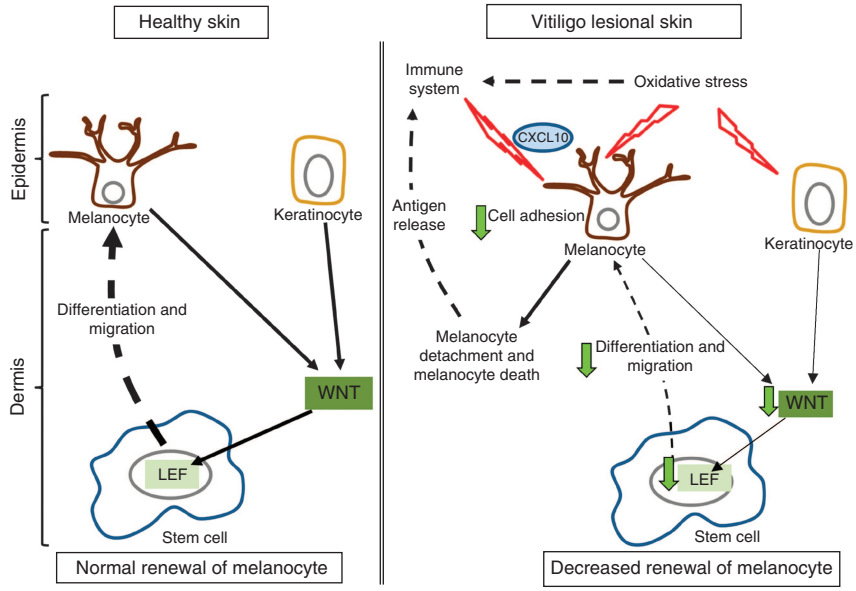


Figure 6. Schematic representation of factors involved in vitiligo pathogenesis. In healthy skin, the stimulation of the WNT pathway by keratinocytes and melanocytes induces the differentiation and proliferation of melanocyte stem cells, allowing the constant turnover of the pools of epidermal melanocytes. In vitiligo skin, oxidative stress can trigger the immune reaction in a genetically predisposed individual. The destruction of melanocytes by the immune system releases melanocyte antigens that stimulate an autoimmune response and ultimately lead to the complete disappearance of melanocyte from the epidermis (and sometimes the hair follicles). Concomitantly, oxidative stress decreases WNT pathway activity in melanocytes and in keratinocytes. This effectively induces decreased cell adhesion with detachment of melanocytes and an impaired differentiation of melanocyte stem cells, altering the capacity of melanocyte turnover. We hypothesize that depending on the patient and the course of the disease, these two mechanisms may be differentially implicated, leading to active depigmentation of the skin and resistance to repigmenting approaches.

(1995) was used for correction of multiple testing. The raw data are available at NCBI GEO, accession number GSE65127.

Functional analysis of gene expression results

Average-linkage hierarchical clustering using Pearson correlation was performed using the Cluster v.3.0 program (Eisen *et al.*, 1998). Graphic outputs were generated with the JavaTreeView3.0 software (Saldanha, 2004). Functional annotation and gene networks for the differentially expressed genes among sample groups were generated using the QIAGEN IPA (QIAGEN, Redwood City, CA, USA; www.qiagen.com/ingenuity).

Ex vivo skin culture

Skin from abdominoplasty surgery was used for the development of the *ex vivo* skin culture model. The subcutaneous fat was removed, and biopsies of 6 mm were taken from skin composed of dermis and epidermis. For vitiligo skin biopsies, after informed consent was obtained and the absence of melanocytes with Wood's lamp was verified, two to three 6-mm biopsies composed of dermis and epidermis were taken from lesional skin ($n = 9$). The biopsies are rapidly placed into a 0.4-μm Transwell chamber (Becton Dickinson, Franklin Lakes, NJ, USA) and maintained under semi-liquid culture conditions in "Skin long-term culture medium" (Biopredic, Saint Grégoire, France). The skin was maintained at 37 °C in a 5% CO_2 atmosphere. The culture medium that was supplemented with forskolin (Sigma-Aldrich, Saint-Louis, MO), LiCl (Sigma-Aldrich), CHIR99021 (Calbiochem, San Diego, CA, USA), or SKL2001 (Calbiochem) was changed every day during the 14-day period.

The methods for Luminex 200 system (Luminex Corporation, Austin, TX) quantitation of cytokines in stratum corneum, cells, quantitative real-time reverse-transcriptase–PCR, and histological analyses are detailed in the Supplementary Methods online.

CONFLICT OF INTEREST

This work was funded in part by Galderma. FJ, CM, MR, BM, PR, CM, YR, DP, and JV are employees of Galderma.

ACKNOWLEDGMENTS

This work was funded in part by a grant from the French Society of Dermatology and by the INSERM. This work was performed in collaboration with Galderma Research. The expert technical assistance of Eve Ferrara, Philippe Grundt, Corinne Menigot, Agnès Perrin and Luigi Russo is gratefully acknowledged. Light microscopy was performed in the C3M Imaging Core Facility (part of Microscopy and Imaging Platform, Côte d'Azur, MICA).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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